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ATTEMPTS TO ABBREVIATE TIME TO END-POINT IN FISH HEPATOCARCINOGENESIS ASSAYS

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ABSTRACT

Attempts to Abbreviate Time to End-Point in

Fish Hepatocarinogenesis Assays

by

John A. Couch and Lee A. Courtney

In recent years, the use of freshwater and marine fishes in carcinogen research and environmental carcinogen monitoring has grown. Data have been produced and published which show the usefulness of fish species as supplemental and complemental tools to traditional mammalian carcinogen research. To enhance the use of fish species and add to the understanding of hepatocarcinogenesis in general, a study to characterize lesion development and abbreviate time to end-point (e.g., early indications of neoplastic change) has been undertaken.

To induce tumor development, adult and juvenile Cyprinodon variegatus were exposed to relatively high sub-lethal concentrations of the potent carcinogenic agent, N-nitrosodiethylamine (DEN). Fish were exposed for several weeks under static conditions to initial DEN concentrations of 55 mg/l, dropping to 30 mg/l at exposure's end. Fish were then transferred to clean flow-through water for holding, observation, and characterization of lesion development. Progression of the hepatic effects of DEN is being followed by periodic sampling for histochemistry, histology, and electron microscopy.

The earliest damage in the liver has been assessed and subsequent progression of lesions is being monitored. Results to date suggest that relatively early foci of altered cells may be identified with traditional morphologic criteria as early as eight weeks following onset of exposure to DEN. Histochemical and ultrastructural early indicators of transformation are under investigation and will be described at this presentation.

Adult and juvenile Cyprinodon variegatus were exposed to relatively high sub-lethal concentrations of the potent carcinogenic agent, N-nitrosodiethylamine (DEN). Fish were exposed for several weeks under static conditions to initial DEN concentrations of 55 mg/l, dropping to 30 mg/l at exposure's end. Fish were then transferred to clean flow-through water for holding, observation, and characterization of lesion development. Progression of the hepatic effects of DEN is being followed by periodic sampling for histochemistry, histology, and electron microscopy. The earliest damage in the liver was assessed and subsequent progression of lesions is being monitored. Results to date suggest that relatively early foci of altered cells may be identified with traditional morphologic criteria as early as eight weeks following onset of exposure to DEN. Histochemical and ultrastructural early indicators of transformation are under investigation and are described.

Introduction

In recent years the use of freshwater and marine fishes in carcinogen research and in environmental carcinogen monitoring has grown [1,2,3,4]. Several advances must be made with selected species in order to make fishes advantageous and more practical as assay subjects. Some of these advances should be: 1) precise characterization of neoplastic end-points and progression in experimentally exposed fishes, 2) abbreviation of length in time needed for risk evaluation of carcinogens or suspect agents in fishes, and 3) correlation of end-points for carcinogen effects in fishes with those in other, more routine test species such as rodents (mammals).

Because we believe that fishes, as a phyletic group, have much to teach us about neoplasia and environmental carcinogenesis, we are studying the experimental induction, progression, and fate of neoplasms in the liver of a marine, coastal fish, the sheepshead minnow (Cyprinodon variegatus). The agent used to induce liver lesions in these studies was N-nitrosodiethylamine (DEN). The sheepshead minnow has been used for many years as a toxicological and carcinogen assay subject in our laboratories [5,6]. The purpose of the present study is to: 1) Characterize liver neoplastic development, and 2) reduce or abbreviate times to end-points in liver carcinogen assays utilizing the sheepshead minnow with histological, ultrastructural, and enzyme histochemical end-points.

Materials and Methods

The sheepshead minnow, Cyprinodon variegatus,was selected as a test organism for this tumor induction and characterization study. A breeding and experimental stock of adult C. variegatus was collected in late April 1983, from the Range Point and Big Sabine areas of Santa Rosa Sound, in Escambia County, Florida. An experimental group of approximately 400 fry

were cultured from this stock and maintained under flow-through conditions until the start of the experimental exposure in mid-October, 1983, at which time the fish were approximately 2 months old. Also, two beginning groups of 30 fish from the April 1983 collection, 15 male and 15 female, were used for control and exposed adult experimental groups.

Exposures were conducted in two glove-box systems, one for the adults, and one for the juveniles, similar to those systems described by Courtney and Couch[6].

N-nitrosodiethylamine (DEN) was selected as a test compound because of its known carcinogenic properties. The test compound was obtained from Sigma Chemical Company * (Cat. # NO258, Lot # 62F01112). The DEN was dissolved in the seawater, then injected into the exposure tanks. The resulting nominal concentration of DEN in each exposure system was 66.7µg/l. DEN concentrations in each test system were monitored using gas chromatography of seawater samples. Initial measured concentrations of DEN in the water column were 55-59µg/l, and gradually decreased during the exposure to approximately 33µg/l at five weeks (Fig. 1).

Exposure of the juvenile fish to the DEN lasted five weeks, at which time the fish were removed from the exposure system, rinsed in several changes of clean seawater, and transferred to flow-through holding facilities for grow-out and observation. Adult fish were exposed for a total of six weeks, then also transferred to flow-through holding tanks.

During the course of the exposure and subsequent holding, periodic samples of fish were taken from each experimental group (exposed and control) for histological, enzyme histochemical, and ultrastructural evaluation.

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* Use of company or trade names does not constitute endorsement by the U.S. Enviornmental Protection Agency.

Scheduled samples were taken at 3, 8, 14, 23, and 28 weeks. In addition, a single juvenile, found in a stressed and disoriented condition at 21 weeks, was sampled for light microscopy only. The experimental design and chronology are diagrammed in Figure 2.

The following four paragraphs outline the procedures for each of the sample types: enzyme histochemical, ultrastructural and histological. Each fish sampled was anesthetized with methane sulfonate (100 mg/1), then dissected and examined grossly. Further processing was performed as follows: The most critical phase of processing with respect to time was the enzyme histochemical procedure; rapid freezing was required for optimum preservation of enzyme activity. Juvenile samples were frozen whole in 2-methylbutane cooled to liquid nitrogen (LN_2) temperature (or directly in LN_2 if 2-methylbutane was unavailable) immediately following anesthesia. Livers from adult fish were separated from the visceral mass, bisected, and then one segment of liver frozen in LN_2 cooled 2-methylbutane. Following freezing, each sample was placed in a LN_2 cooled Nunc vial, labeled and stored in an ultra-low temperature freezer at -80°C until shipment to Dr. David Hinton at the Medical Center School of Medicine, Morgantown, West Virginia, for enzyme histochemical analysis and evaluation. In Morgantown, samples were stored in LN_2 until histochemical processing could begin.

Prior to processing for glucose-6-phosphate dehydrogenase (G6PD) activity, tissue samples were allowed to equilibrate in a cryostat at -15°C for 10 minutes. Fresh frozen cryostat sections (10μ) were then reacted for G6PD using a formazan method[7]. Additional methods are being evaluated are; glucose-6-phosphatase (GGP), adenosine triphosphatase (ATPase), and gammaglutamyl transpeptidase (GGTP). These will be reported at a later date.

For electron microscopy processing a small piece of tissue from the liver of either juvenile (with liver still in visceral cavity) or adult fish (unfrozen half of bisected liver) was placed in a 3% solution of gluteraldehyde (GTA) in 0.1M Millonig's phosphate buffer (pH 7.4) and minced into small (approximately 1 mm^3) pieces. Following approximately 1 hour GTA fixation, the tissue was then post-fixed in a 1% solution of osmium tetroxide (OsO_4) for 4-5 hours[8], rinsed overnight in phosphate buffer, then dehydrated in a series of acetone solutions, and embedded in Epon 812. Sections were cut at 500-600 A, stained with uranyl acetate and lead citrate[8], and examined for ultrastructural pathology.

Samples for light histology were fixed in Davidson's fixative for 24-48 hours. Adult livers were processed as discrete samples. Visceral masses were also processed and embedded from each adult fish. Juvenile fish were processed whole. Sections were cut at 5 μ and slides stained with a standard Harris' hematoxylin and eosin procedure[9].

RESULTS

The incidence of microscopic toxic, preneoplastic, neoplastic, and neoplastic-like lesions is given for both exposed adult and juvenile *C. variegatus* in Table 1. A spectrum of toxicity-related lesions and at least three probable neoplasms were found in fish samples between 3 and 28 weeks following onset of exposure to the DEN (Table 1). We will describe these lesions in the chronology of their appearance in the experimental fish.

Following the description of the induced lesions and their chronology, we will suggest, based on evidence, a progression of lesions from toxic to preneoplastic to neoplastic.

Responses in livers of fish sampled at three and eight weeks.

Control fish examined during the study all had normal livers based on gross and microscopic examinations. For comparison with lesions hereafter described, a brief description of the normal liver of C. variegatus, typical of most teleost fishes, is provided here.

The liver parenchyma (Fig.3) is composed of hepatic cells of fairly uniform size and shape. Architecturally, the hepatocytes are arranged in muralia, generally two cells thick, along blood sinusoids with bile canuliculi and ductiles, leading to bile ducts, between the cell layers. Nuclei are homogeneous in size, generally round, and fairly regular in shape, with a single, centrally located nucleolus.

Experimental juvenile and adult livers in three and eight week samples (Table 1) demonstrated the most severe toxic cellular and histologic responses. Microscopic examination revealed that hepatocytes were slightly to dramatically enlarged, rounded, and without visible muralia or cord relationship to one another (Fig.4). Throughout liver sections there were foci and bands of lymphocytes and fibroblasts, and granulomata were found where cell death and necrosis were evident (Fig. 5). In some livers from adults, small islets of dying hepatocytes were obvious and early indicators of cirrhotic change (fibrous tissue being deposited) were evident. Presumed ceroid deposits were abnormally abundant in the eight week adult livers. Clear cell areas (see Figs. 7A and 7B) were found in focal and trabecular patterns in some adult and juvenile liver sections (Table 1). The toxic effects of the DEN exposure appeared similar in the three and eight week samples, but were more severe in the adult fish than in the juveniles.

With light microscopy, some possible preneoplastic foci were found in at least two adult fish from the eight week sample (Table 1). These foci

consisted of two apparent cell types: larger, possibly altered hepatocytes arranged as disorganized nodules, and smaller spindle to oval cells of uncertain origin (Figs. 6A and 6B). Both cell types were pleomorphic, displaying cytomegaly and karyomegaly, and showed evidence of mitotic activity. In both three week and eight week livers, occasional eosinophilic foci or widespread eosinophilia (of less distinct limits) were noted.

Most electron microscopic (EM) findings in the three and eight week samples probably reflected the intoxicated state of hepatocytes rather than distinct preneoplastic changes although a few changes in the exposed fish hepatocytes may relate to early neoplastic alteration. The better EM results were obtained from the eight week adults. Sections from control livers revealed expected, normal hepatocyte profiles. Plasmalemma were distinct, nuclei were round to oblong, and endoplasmic reticulum (ER) was distributed in a perinuclear configuration with large lakes of glycogen occupying most of the cell.

The exposed liver sections possessed alterations as follow: nuclei were slightly irregular in profile in comparison with control specimens. Some nuclei were severely lobulated with intrusions of cytoplasm appearing as bay regions within nuclei or as pseudo-inclusions (membrane-bound nuclear inclusions). Cytoplasm, in general, was diffusely spread throughout the cell. Mitochondria were enlarged and the ER in the cytoplasm was diffuse and lacked organization. The glycogen content was moderate to light and lipid content minimal. Tonofibrillar material was abnormally common. Bile canuliculi appeared normal.

To date, enzyme histochemical analysis of samples taken during the exposure and holding phases of the study are underway but incomplete. Early indications show promise for the G6PD reaction. Control livers of

both juvenile and adult Cyprinodon displayed a diffuse, moderate, blue reaction over hepatocyte cytoplasm throughout all regions of the sections. By contrast, areas of toxic response in liver sections exposed to DEN reacted with less intensity than that of control liver. By 8 weeks rounded foci of intense reactivity characterized liver sections from some individuals. The enzyme alterations evidenced by this reaction and that of 3 other enzymes, commonly employed in mammalian liver carcinogenesis studies, suggest a progressive pattern of enzyme activity alteration in the development of liver lesions in Cyprinodon. This sequence is under investigation to determine its relationship to overt liver tumors. It may be possible to use early enzyme altered foci to determine the role of specific populations of cells in tumor development and to possibly decrease the time between exposure and end-point (early altered foci).

Responses in livers of fish samples at 14 weeks

Microscopic evidence of toxic effects and inflammatory responses (Table 1) persisted in juvenile and adult livers sampled at 14 weeks. However, the general view indicated that livers in both groups were regenerating, and resorbing necrotic cell material, while moderating the inflammatory response described for the three and eight week samples (less massive infiltrates or foci of lymphocytes, fibroblasts, or macrophages). There were, however, very abundant and extensive abnormal deposits of ceroid-like and hemosiderin-like materials in all the livers from this sample. This was probably related to the prolonged intoxication of the liver during the exposure of several weeks.

Clear cell foci (7A and 7B) were observed in most of the juvenile and adult exposed fish samples at this time. These clear cell foci consisted of hepatocytes in a focal or quasi-trabecular pattern. The clear cell

hepatocytes (Fig.7A) each had a clear to lacy cytoplasm with a distinct, centrally located nucleus, resembling closely the preneoplastic clear cell foci described by Frith and Ward[10] for livers of mice exposed to a variety of chemical hepatocarcinogens. Additionally, some clear cell areas were noted consisting of cells with large vacuoles and nuclei displaced to the periphery of the cell (Fig.7B). Similar areas, referred to as vacuolated cell foci, were also noted by Frith and Ward[10] in rats undergoing hepatocarcinogenesis.

Generally, among most of the livers examined from this sample, the most striking histopathological characteristic was the considerable pleomorphism expressed among hepatocytes. Cytomegaly, karyomegaly (3-5 x enlarged cells & nuclei), and nuclear and cytoplasmic inclusions (eosinophilic and basophilic) were common features. Mitoses were more common in these hepatocytes than in those of the three and eight week samples or in those of any of the control fish livers examined. Many of the pleomorphic, enlarged cells were arranged in trabecular or nodular foci and may represent centers of early neoplastic hepatocytes.

One of three juveniles examined from the 14 week samples had advanced trabecular hepatocellular carcinoma (Figs. 8A and 8B). This neoplasm occupied most of the liver and was characterized by cellular pleomorphism, loss of any normal liver cord architecture, cytomegaly and karyomegaly of hepatocytes, some multi-nucleate cells and very high mitotic activity with abundant abnormal (tripolar; polyploid) mitoses (Fig. 8A). Additionally, cholangiolar transformation and proliferation had occurred in this liver, producing mixed, hepato-cholangiolar carcinoma.

Electron microscopy of these samples revealed the most striking differences between control and exposed hepatocytes to date. Control hepatocyte (Fig. 9) nuclei were normal in profile, appearing round to

oblong. The cytoplasm was also normal in appearance, with small to large lakes of glycogen, and a perinuclear to diffuse distribution of organelles. Most hepatocytes had extensive rough ER, visible Golgi apparatus, and normal mitochondria (round to elongate tubular forms with shelf-like cristae). Only modest lipid inclusions were noted in the hepatocytes, and the plasmalemma appeared normal. Intercellular relationships, sinusoids, endothelium, and bile canuliculi were normal.

Exposed hepatocytes (Fig. 10) generally had nuclei that were more irregular in profile. Nucleolar organization was altered (more condensed) and bays or invaginations of cytoplasm into the nucleus were observed. The cytoplasm was without normal lakes of glycogen in most instances. Rough ER was less abundant and considerably more dilated; smooth ER was present. Phagolysosomes were abundant in the cytoplasm. Most mitochondria were abnormally swollen and were round to globose in appearance. Only moderately distributed lipid inclusions were found. Plasmalemmae were difficult to distinguish for many hepatocytes, and bizarre folding and interdigitation of the plasmalemma was obvious. Little intercellular space was visible and cells appeared to be swollen or enlarged. Significant amounts of tonofibrillar material were noted within cells (Fig. 10). Recent reports describe similar tonofilaments in human carcinomas of the gall bladder[11]. Bile canuliculi were difficult to discern (occluded).

Pathology of liver of single moribund fish examined at 21 weeks.

A single, exposed juvenile fish became disoriented and moribund, but was taken alive, fixed and processed for histopathology at 21 weeks from the onset of the exposure (Table 1). The condition of the liver of this specimen was so remarkable that a section devoted to it here is deserved.

Grossly, the liver appeared very abnormal. It was pale white in color

with apparent, small, firm nodules beneath its capsule. Microscopically, all liver sections from this fish showed advanced neoplasia. Two major neoplastic cell types were evident. First, transformed hepatocytes made up nodules and trabeculae that occupied 50% to 60% of the liver (Fig. 11A). These cells and nodules probably represent advanced hepatocellular carcinoma in which all normal liver cord architecture was lost. The second transformed cell type appeared to be ductile in origin and formed disorganized or degenerate ductile and tubular elements in the remaining 40% to 50% of the liver sections (Fig. 11B). These cells were oval to oblong, and only one-fourth to one-half the size of the neoplastic hepatocytes. These cells probably constituted cholangiolar carcinoma. Both cell types had extensive mitotic activity indicating continued proliferation.

Most likely, this fish was dying because of liver failure resulting from almost complete loss of normal liver structure and function.

Responses in livers of fish samples at 23 weeks.

Grossly, the livers of adults from this sample appeared muddy brown and mottled with white. The livers of the juveniles appeared red to beige. Both groups of livers had abundant pigmented macrophage centers beneath the capsule. These foci appeared as black splotches or spots and were absent in control fish livers.

Microscopically, the majority of the liver parenchyma had recovered from the toxic effects in both adults and juveniles. There were, however, certain residual indicators of cytotoxic reaction in the sections. Spongiosis hepatis lesions were evident in most livers (Fig. 12). These lesions may result from foci of necrotic cells that had been injured by the direct toxic effects of the DEN. To our knowledge, this lesion has not been described in fishes; however, it is similar to lesions described in mammalian

species exposed to carcinogens.[12] Occasionally, granulomata were found in the liver sections, also indicating residual repair and clean-up of necrotic foci by encapsulating and phagocytic cells.

Preneoplastic development was suggested by the presence of distinct clear cell foci and eosinophilic foci.

Adult livers revealed overtly developing hepatocellular and cholangiolar carcinomas. The hepatocellular neoplasms were both nodular (Fig. 13) and trabecular (Fig. 14). The most striking findings in these sections were several neoplastic foci of mixed cells,both hepatocytes and ductile cells. The cholangiolar cells were arranged in sinuous strands projecting from central foci into surrounding areas of neoplastic and more normal hepatocytes (Fig. 14). Livers of juveniles sampled at this time revealed suspect atypical nodular and trabecular foci but did not have advanced neoplasia (Table 1).

Responses in livers of fish samples at 28 weeks.

The majority of livers from exposed juvenile and adult fish taken at 28 weeks had either incipient or advanced (or both) neoplasms. At least three kinds of neoplasms were tentatively identified in the livers of these fish (Table 1).

Hepatocellular carcinoma, cholangiolar carcinoma, and hemangiosarcoma were found in two of three adult livers. Juveniles suffered from hepatocellular carcinoma and cholangiolar carcinoma. The occurrence of apparent and clearly differentiated hemangiosarcoma in livers of exposed fish was first noted in this sample (Table 1). Both trabecular and nodular hepatocellular carcinoma, and cholangiolar carcinoma were observed in sections of adult livers and trabecular hepatocellular and cholangiolar carcinoma in juveniles.

The presumed hemangiosarcomas were observed in adult livers, and will be described in some detail. At lower magnification (x 125), invasive,

anastomosing tracts of probable blood vessel wall cells were apparent in areas of the liver (Figs. 15A and 15B). These tracts had their origins in the walls of large blood vessels in the livers (Fig. 16A). The cells that composed these tracts were oval to elongate to spindle-shaped and were actively proliferating (Fig. 16A). The nuclei of these cells were similar in size and form to those of normal endothelial cells (Fig. 17) in the hepatic blood vessels from which they apparently arose.

The invasive tracts formed by these cells in some cases gave rise to large pseudo-vascular regions (Figs. 15B and 16B). The cells making up these pseudo-vascular-like regions often assumed a squamous form and formed incomplete endothelia of blood vessels (Fig. 16B).

Examination of other tissues revealed no evidence that any of the tumor types described had metastisized.

Discussion

The fate of these liver lesions is being monitored in the remaining fish in this experiment and will be reported in another paper. The observations and data gathered to date, however, permit us to suggest possible trends in the progression of the lesions through the first 28 weeks of their development. Comparison of the development of the lesions in C. variegatus has been made with occurrence of chemically induced liver lesions in mice as described by Frith and Ward[10], in rats [13, 14, 15], and in rainbow trout as described by Hendricks[16]. Further comparisons of the sheepshead minnow lesion development have been made with the cellular lineage model proposed for experimental hepatocellular carcinoma in rats by Sell and Leffert[17].

The earliest detectable, probable preneoplastic lesions occurred between 8 and 14 weeks following onset of exposure. These initial indicator

lesions were clear cell foci and atypical eosinophilic, and more rarely, basophilic nodules or trabeculae (Figs. 6A, 6B, 7A, and 7B). The first overtly microscopic neoplasms were trabecular and nodular hepatocellular carcinomas (14 week sample and 21 week individual: Table 1) and cholangiolar carcinoma (14 week sample and 21 week individual: Table 1). Indeed, when examined in detail, it is quite possible that these neoplams were actually mixed hepato-cholangiolar carcinomas similar to those induced in rat livers with DEN and described by Schauer and Kunze[13].

It is interesting to compare the hemangiosarcomas induced in rat liver by Schauer and Kunze[13] with DEN to those just described herein induced with DEN in C. variegatus liver. The cytology and histology of these hemangiosarcomas in rat liver and sheepshead minnow both consist of spindle-shaped to ovoid cells arranged in anastomosing networks or tracts (Figs. 15A and 15B).

Enzyme histochemistry being conducted as part of this study is far from complete, however, as mentioned in the results section, results, particularly with G6PD, offer promise for possible detection of early altered, transformed foci of induced liver neoplasmas in C variegatas. This work will continue and will be reported later.

As stated earlier, not all of the electron microscopy results are analyzed yet; in fact, the samples from the livers with more advanced tumors are still under evaluation. However, distinct ultrastructural differences between exposed and control tissues have been noted in the earlier samples. Most of the differences are possibly related to cytotoxic effect of the DEN exposure (i.e., reduced glycogen reserves, swollen mitochondria, and ER alterations). Some changes, for example, alterations of nuclear morphology (lobulation of nucleus, alteration of chromatin material, and nuclear inclusions), plasmalemmal alterations and drastic increase in tonofibrillar

elements may be related to preneoplastic and neoplastic changes. More investigation into the significance of the noted ultrastructural changes and examination of unfinished samples is presently underway.

Though the timing of appearance of specific microscopic liver lesions in sheepshead minnows differs somewhat from that in rodents, the sequence of lesion types, ranging from toxic to preneoplastic to neoplastic, is generally similar to that proposed by Sell and Leffert[17] for the rat. The sheepshead minnow sequence resulting from relatively high dose exposure (50-60 µg/1) to DEN is:

General Cytotoxic Response
(3-8 wks)
Figs: 4,5,and 12

Clear Cell Foci
Nodular Foci and Trabeculae
(8-14 wks)
Figs: 6A,6B,7A,and 7B

Hepatocellular and Cholangiolar Carcinoma, Mixed Cell Hepato-Cholangiolar Carcinoma, and Hemangiosarcoma
(14-28 wks)
Figs: 8A, 8B, 11A, 11B, 13, 14, 15A, 15B, 16A, and 16B

Whether or not the sequence of lesion development that is described here for C. variegatus as induced by DEN is representative of what occurs in other fishes exposed to a variety of chemical agents is uncertain. Indeed, the sequence of events for chemically induced liver lesions leading to neoplasia for rainbow trout[16], exposed to the more potent hepatocarcinogen, Aflatoxin B_1, is more discrete and perhaps less complex than that described herein for the sheepshead minnow, even though time for lesion development is longer in the cold water rainbow trout.

The response of the sheepshead minnow to the known carcinogen, N-nitrosodiethylamine, relegates this species to the useful category of

small fish species available for carcinogen monitoring and assays.

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Figure Legend

Fig. 1 Concentration of N-nitrosodiethylamine (DEN) in water column of static test systems (adult and juvenile) during exposure phase of tumor characterization study.

Fig. 2 Gant chart showing experimental design and schedule.

Fig. 3 A control liver from an adult C. variegatus detailing hepatocyte-sinusoid cord pattern. The cells are generally cuboidal, and nuclei relatively consistent in size, containing a single centrally located nucleolus. X 1875

Fig 4 Hepatocytes in exposed adult liver, sampled at 8 weeks, displaying severe toxic response. Muralia are indistinguishable, cytomegaly and karyomegaly (K) evident; binucleate cells (B) and nuclei with multiple nucleoli (N) are present. X 2500

Fig 5 Granuloma in liver of 3 week juvenile fish. Foci of necrotic cell material, resulting from cytotoxic effects, is surrounded and encapsulated by basophilic cells consisting of lymphocytes, macrophages and fibroblast-like cells. X 1875

Fig. 6A and 6B

Liver sections of 8 week adult C. variegatus which show possible preneoplastic nodule formations. Nodules are composed of altered hepatic cells (H), highly pleomorphic in nature, surrounded by smaller sinuous cell type (S) also displaying pleomorphism. The nuclei of the hepatic cells vary greatly in size and shape (karyomegaly) and are generally larger than those of the second cell type. Also note the loss of normal liver cord pattern. A: X 1875, B: X 1875

Fig. 7A and 7B

Examples of clear cell areas found in specimens from most samples. A: Clear cell foci consisting of cells with clear to lacy cytoplasm and centrally located nuclei. Similar clear cells were also noted in more diffuse trabecular patterns. B: Vacuolated clear cell area in which large lipid droplets displace the nuclei to the periphery of the cell and give the cell a clear appearence. A: X 1875, B: X 1875

Fig. 8A and 8B

Advanced hepatocellular carcinoma found in juvenile C. variegatus sampled at 14 weeks. Note extreme pleomorphism of neoplastic hepatocytes, cytomegaly and karyomegaly. Binucleate cells (B), abnormal nuclei (A) containing multiple nucleoli and inclusions, and abnormal division figures (M) were found throughout the entire liver from this specimen. A: X 1875, B: X 2500

Fig. 9 Electron micrograph of control adult liver showing typical ultrastructure of hepatocytes. Note lakes of glycogen (G), extensive, highly organized rough ER (ER), and appearance of normal mitochondria (NM). X 5,000

Fig. 10 Electron micrograph of experimental adult liver (14 week) showing alterations in ultrastructure relating to DEN exposure. Note irregular lobulation of some nuclei, almost total lack of glycogen, greatly reduced rough ER and considerable increase in dilated smooth ER lacking organization seen in controls, and swollen mitochondrias. Unusual folding and interdigitation of plasmalemma (BP) and fairly large amounts of tonofibrillar material (T) were noted in exposed liver sections. X 5,000

Fig. 11A and 11B

A: Liver of individual sampled at 21 weeks showing advanced trabecular

and nodular hepatocellular carcinoma occupying approximately 50% to 60% of the hepatic parenchyma. B: Discrete hepatocellular and cholangiolar elements are present throughout this specimen, however, areas of mixed cell neoplasia (hepatocholangiolar carcinoma) are also evident. A: X 625, B: X 2500

Fig. 12 Spongiosis hepatis lesion from adult exposed fish sampled at 23 weeks. Lesions of similar nature were found in other specimens from the 14, 23, and 28 week samples. X 625

Fig. 13 Nodular hepatocellular carcinoma at 23 week sample. X 1875

Fig. 14 Mixed cell neoplasia at 23 weeks, consisting of cholangiolar (CC) and trabecular hepatocellular (HC) elements. X 1875

Fig. 15A and 15B

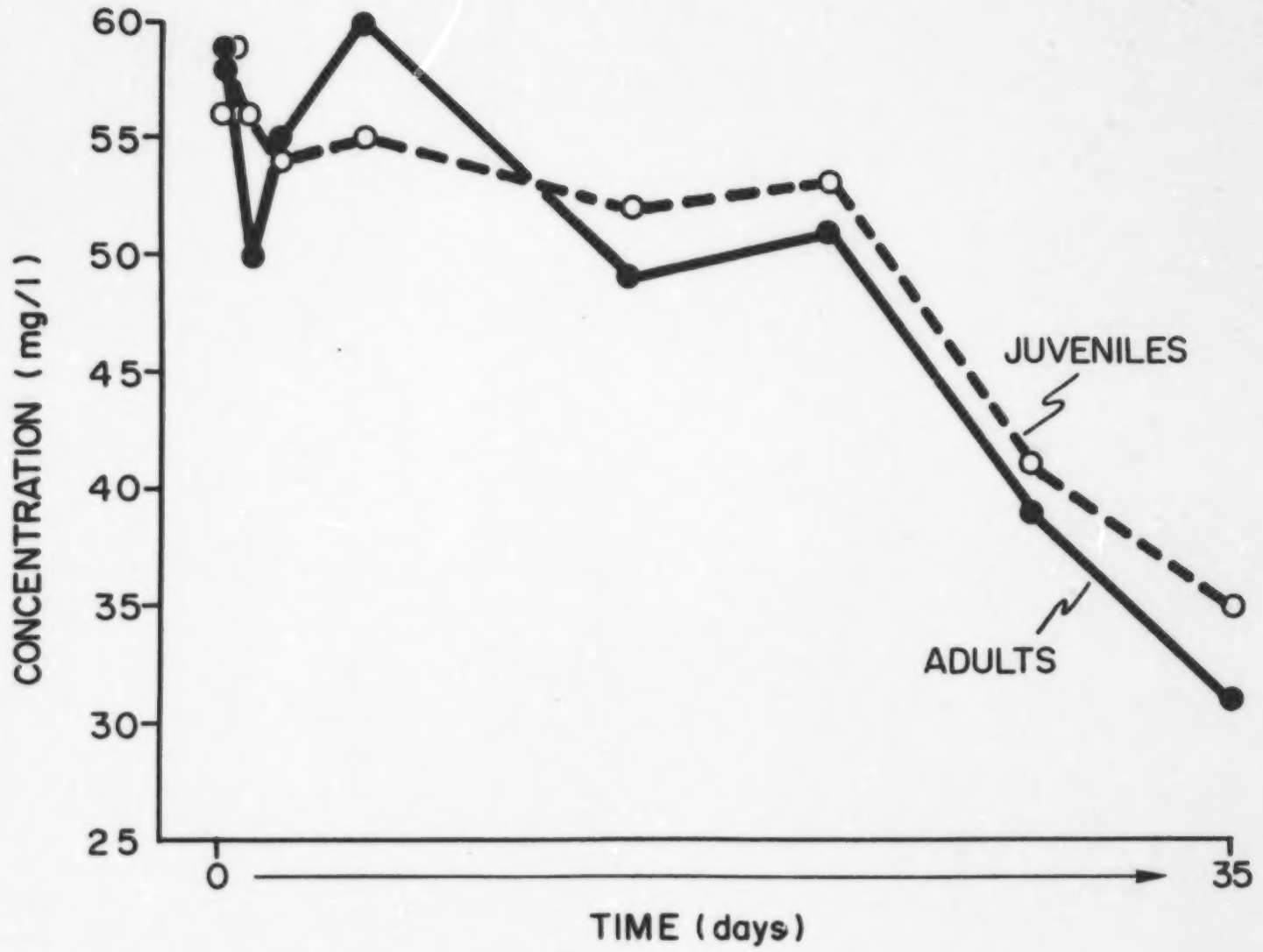
Hemangiosarcoma (HS) observed in adult liver from 28 week sample. Invasive anastomosing tracts (T) run through liver parenchyma, composed of elements of hepatocellualr and cholangiolar carcinoma. Origin of neoplasm appears to be from major blood vessel (V) walls. Note pseudo-vascular region (PV) formed by proliferating cells assuming a squamous form. A: X 625, B: X 625

Fig. 16A and 16B

A: Proliferating endothelial lining of hepatic blood vessels from 28 week exposed adult fish. High mitotic activity is indicated by division figures (M). Cells are oval to elongate to spindle-shaped, with nuclei similar to normal blood vessel endothelial cells (see Fig. 17). B: Pseudo-vascular region of hemangiosarcoma from exposed adult sampled at 28 weeks. Note squamous form of the cells (S) forming abnormal, incomplete blood vessels (IV). Pseudo-vascular region depicted here are surrounded by actively proliferating (M: mitotic figures) elements of hepatocellular

carcinoma (HC). A: X 1875, B: X 1875

Fig. 17 Endothelial cell lining of blood vessels and sinusoids in the liver of control C. variegatus. Note the normal one to two cell thickness of vessel wall. X 2500


60
55
50
45
40
35
30
25
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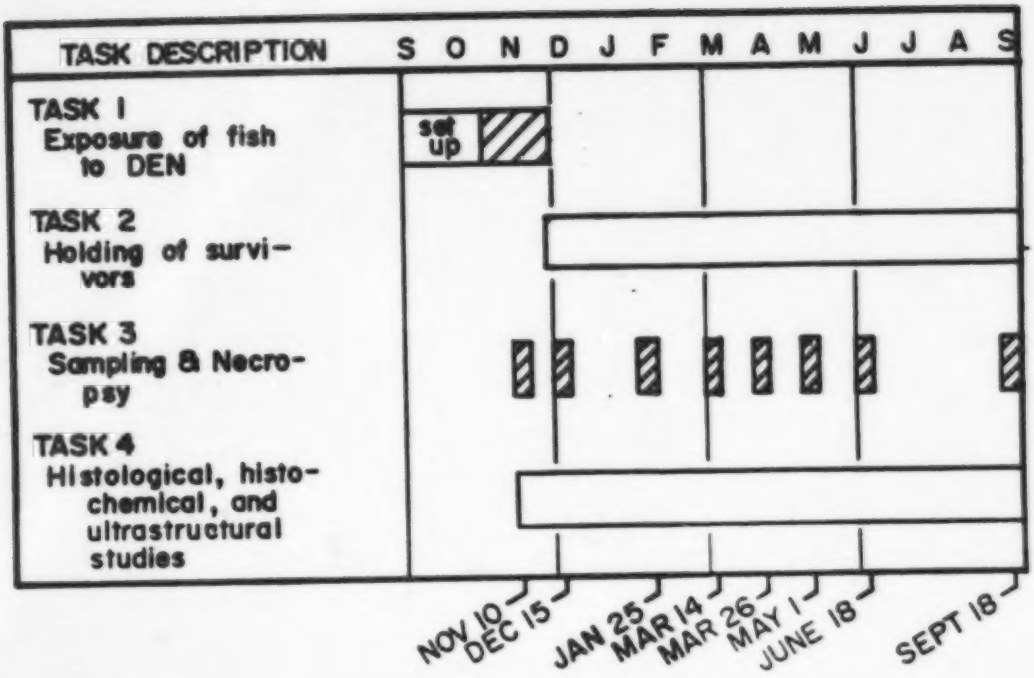
JUVENILES
ADULTS
0
35
TIME (days)


TASK DESCRIPTION
S O N D J F M A M J J A S
TASK 1
Exposure of fish to DEN
set up
TASK 2
Holding of survivors
TASK 3
Sampling & Necropsy
TASK 4
Histological, histochemical, and ultrastructural studies
NOV 10
DEC 15
JAN 25
MAR 14
MAR 26
MAY 1
JUNE 18
SEPT 18

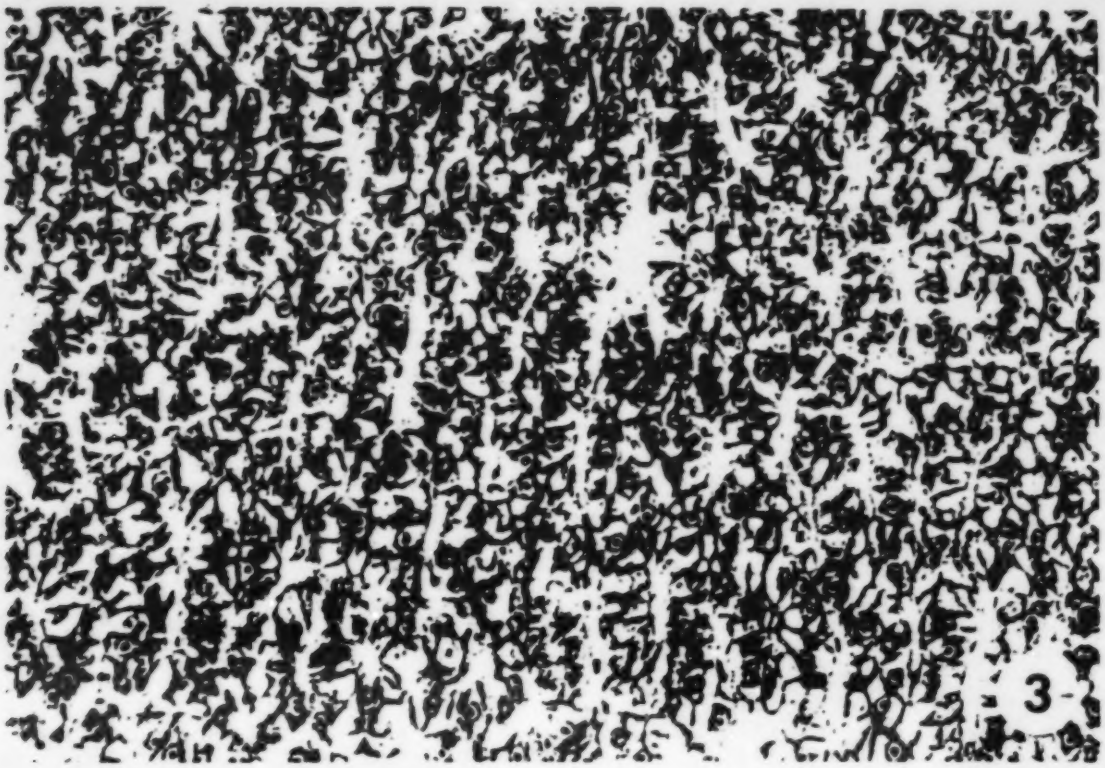
OBSERVATION PERIOD, wks.	MICROSCOPIC ALTERED FOCI OR LESIONS													
	CYTOTOXIC AND INFLAMM.		CLEAR CELL		ATYPICAL NODULAR OR TRABECULAR FOCI		TRABECULAR[1] HC[2]		NODULAR[1] HC[2]		CHOLANGIOLAR CARCINOMA		HEMANGIO-SARCOMA	
	J	A	J	A	J	A	J	A	J	A	J	A	J	A
3	9/9	4/4	1/9	0/0	0	0	0	0	0	0	0	0	0	0
8	2/2	4/4	2/2	3/4	0	2/4	0	0	0	0	0	0	0	0
14	3/3	3/3	2/3	2/3	2/3	2/3	1/3	0	0	0	1/3	0	0	0
21[3]	0	-	0	-	0	-	1/1	-	1/1	-	1/1	-	0	-
23	4/4	4/5	3/4	4/5	3/4	4/5	0	3/5	0	1/5	0	3/5	0	0
28	3/3	2/3	3/3	2/3	1/3	1/3	2/3	2/3	0	1/3	2/3	2/3	0	2/3

1 SOME OF THESE CASES ARE MIXED CELL CARCINOMAS

2 HEPATOCELLULAR CARCINOMA

3 SINGLE JUVENILE FISH IN DISTRESSED CONDITION WHEN SAMPLED

Table 1. Incidence of microscopic toxic, preneoplastic, neoplastic, and neoplastic-like lesions in livers of sheepshead minnows (Cyprinodon variegatus) experimentally exposed to 50 ug/l N-nitrosodiethylamine for five (juvenile) and six (adult) weeks followed by holding in flow-through systems.



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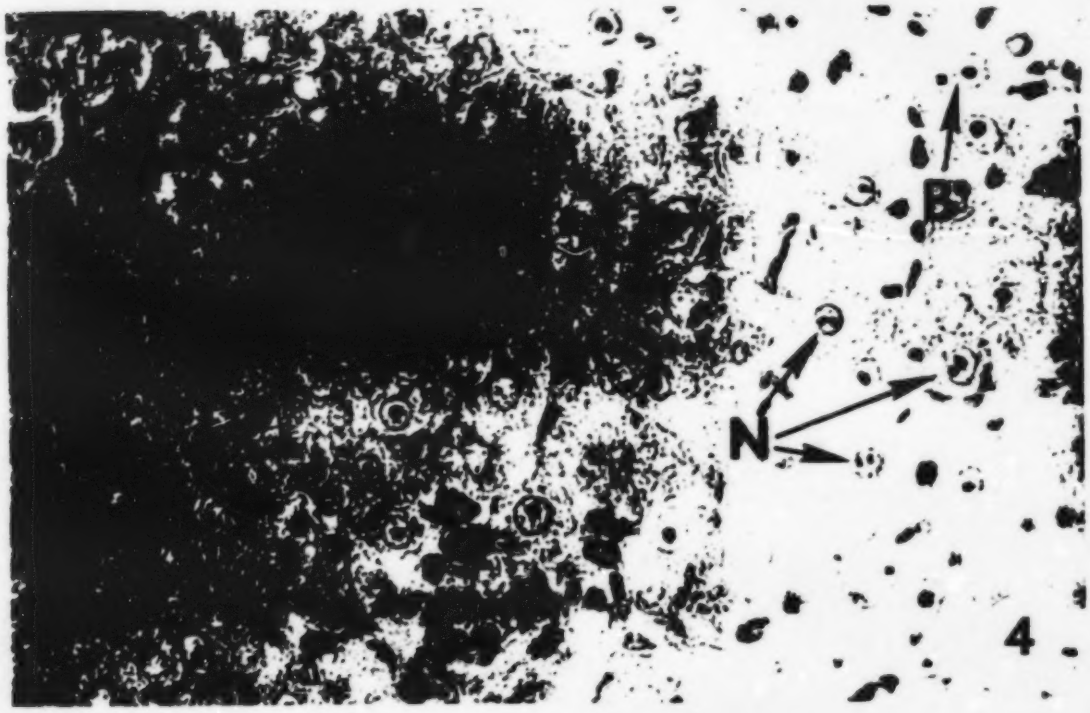

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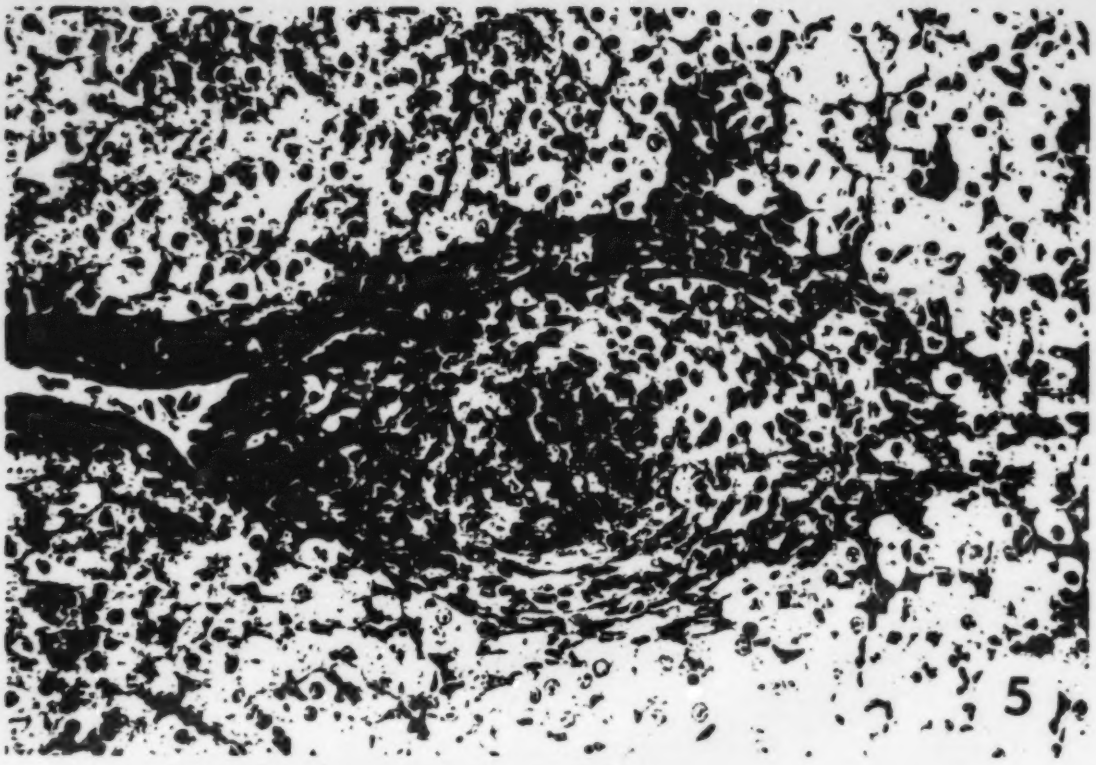
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7A
7B

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G
9

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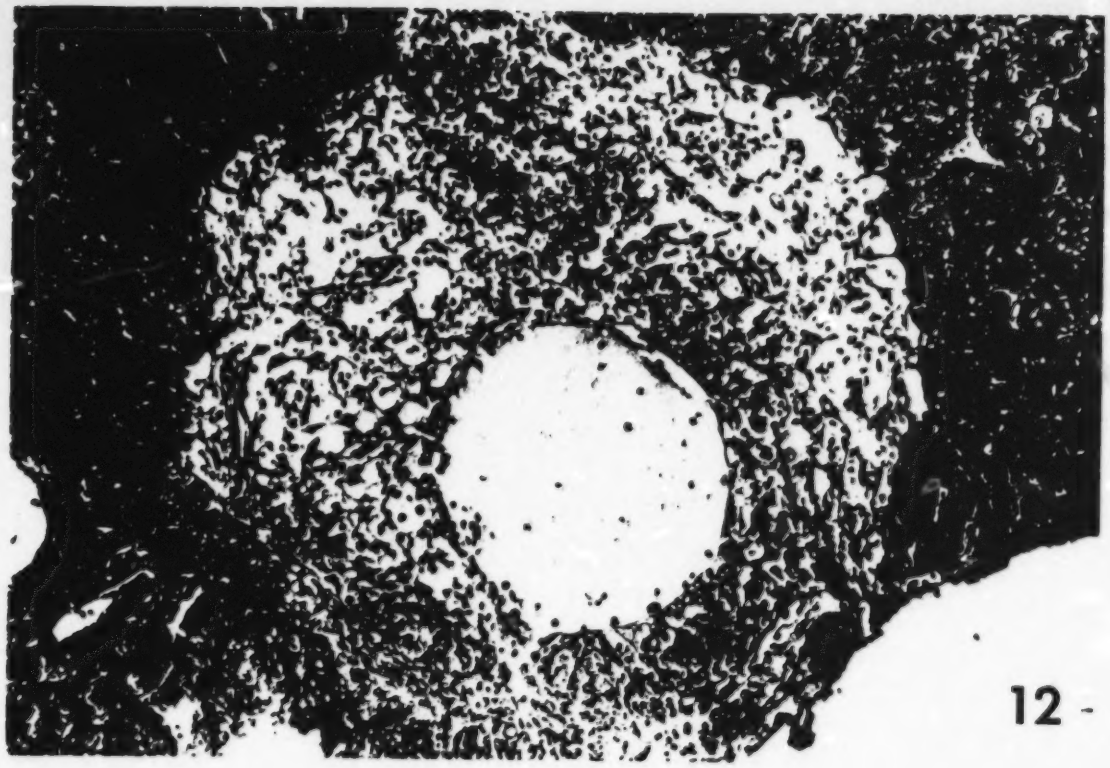
11A
11B



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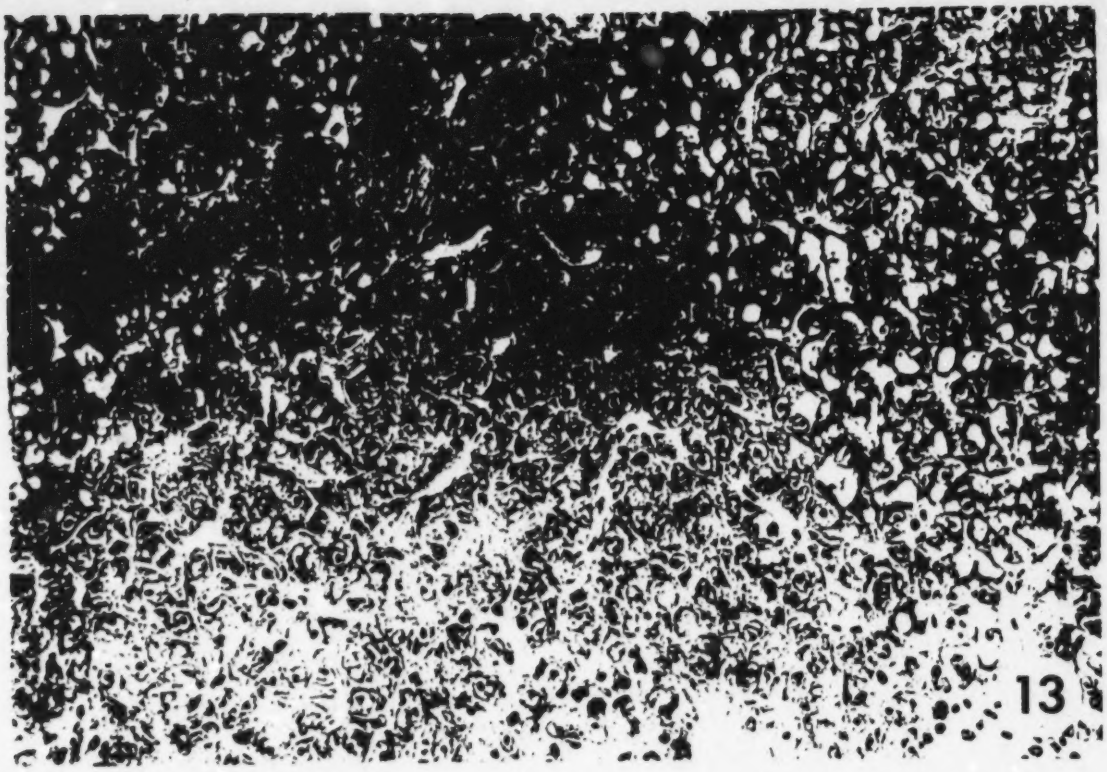

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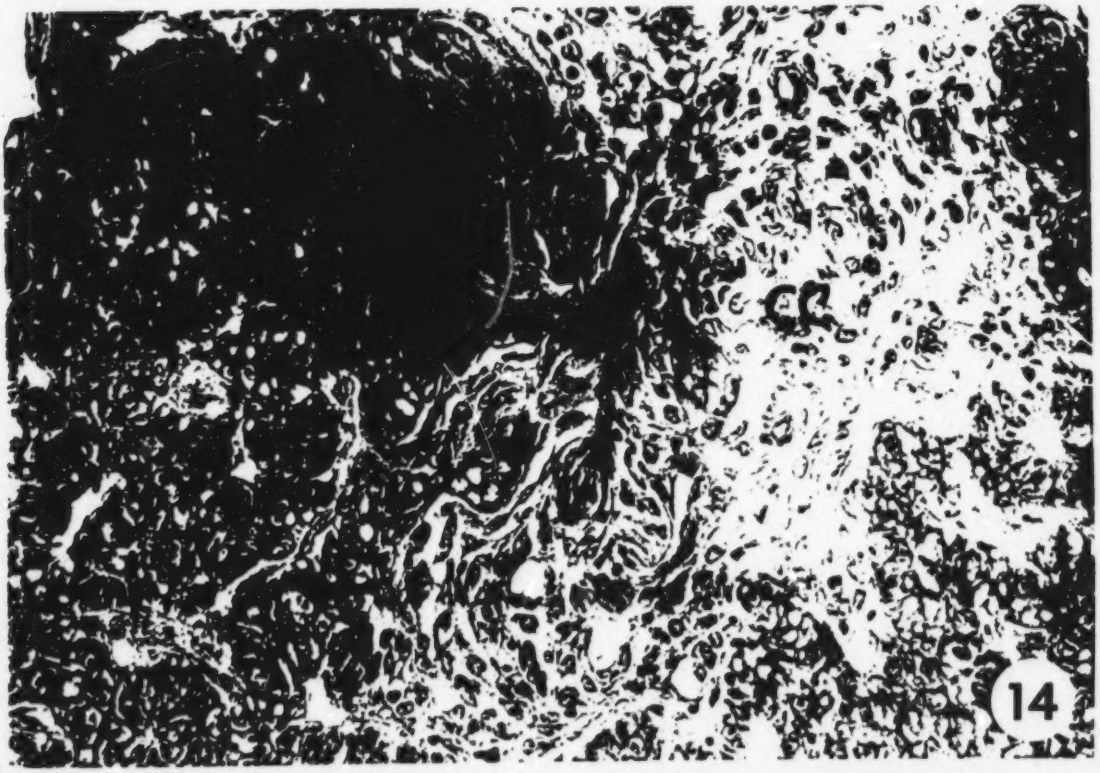

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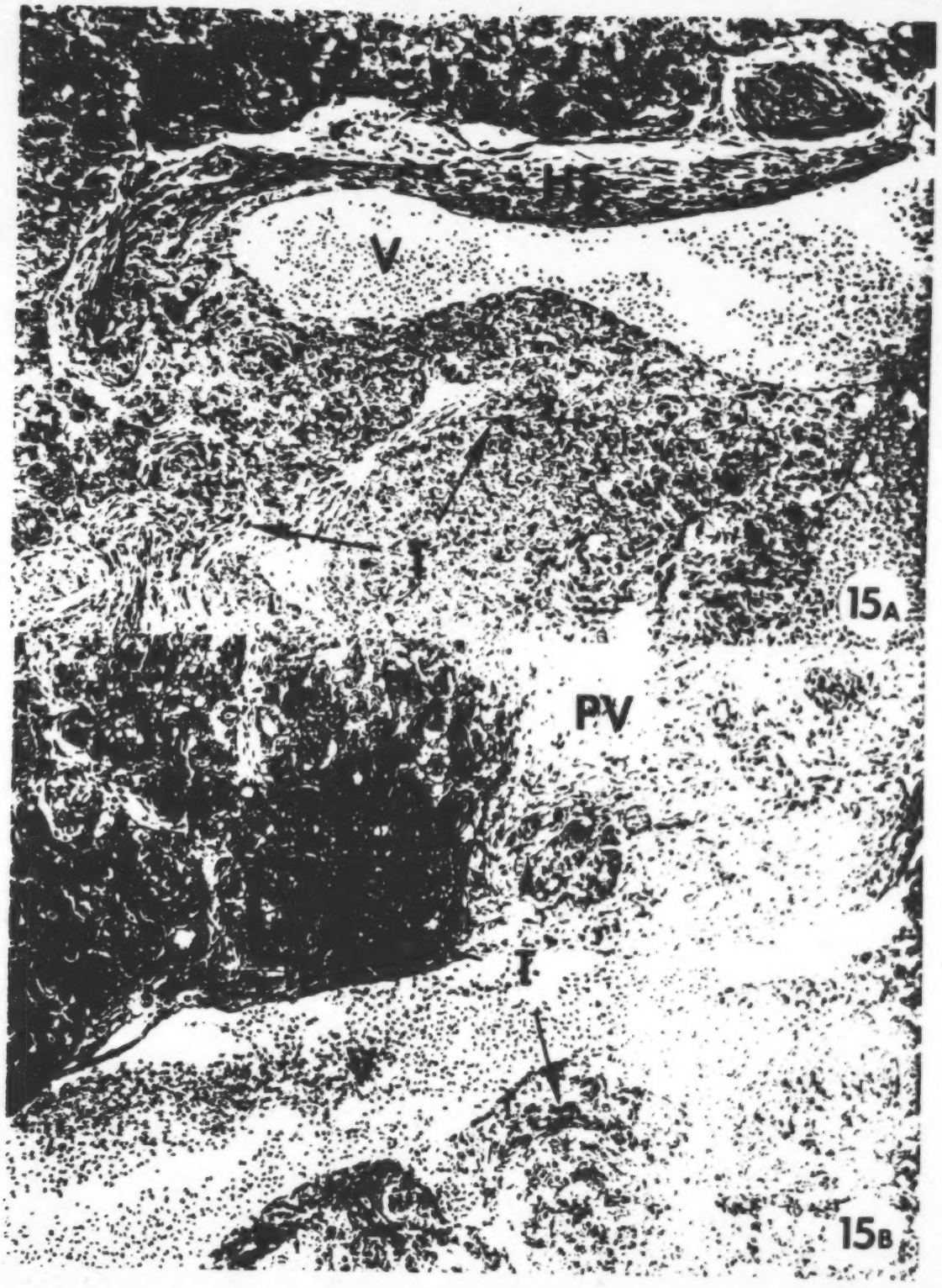

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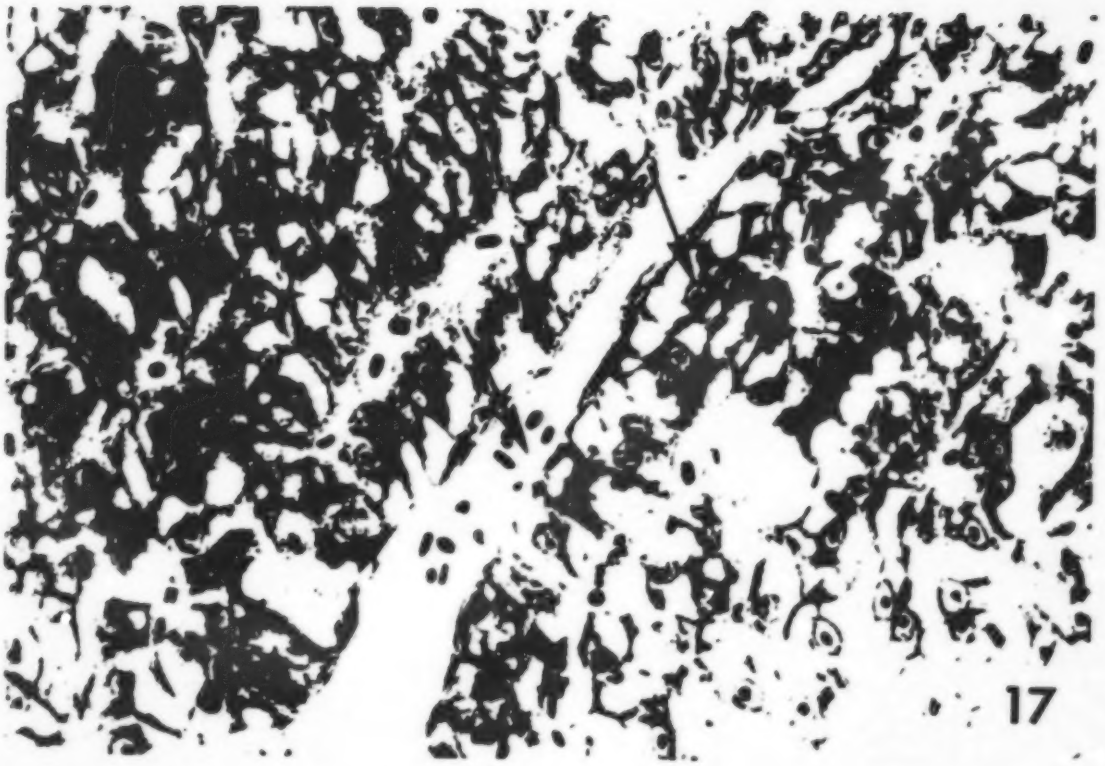
16A
IV
16B



17

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